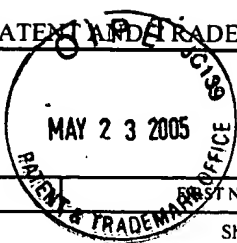




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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/741,843	12/22/2000	Shui-on Leung	018733-0996	9659

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EXAMINER

SCHWADRON, RONALD B

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1644

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Commissioner for Patents

The reply filed 1/13/2005 is not fully responsive to the Office communication mailed 12/16/2004 for the reason(s) set forth below or on the attached Notice To Comply With The Sequence Rules or CRF Diskette Problem Report.

Regarding applicants comments, the "KDEL" in PE38KDEL stands for the amino acid sequence "KDEL" (see Brinkmann et al., page 8617, second column, line 14).

Since the above-mentioned reply **appears to be bona fide**, applicant is given a TIME PERIOD of **ONE (1) MONTH** or **THIRTY (30) DAYS** from the mailing date of this notice, whichever is longer, within which to supply the omission or correction in order to avoid abandonment. EXTENSIONS OF THIS TIME PERIOD MAY BE GRANTED UNDER 37 CFR 1.136(a).

Ron Schwadron, Ph.D.
Primary Examiner
Art Unit 1644


RONALD L. SCHWADRON
PRIMARY EXAMINER
GROUP 1644 (b2)

Notice to Comply

Application No.

09/741843

Applicant(s)

Leung et al.

Examiner

Ron Schwadron,
Ph.D.

Art Unit

1644

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Applicant must file the items indicated below within the time period set the Office action to which the Notice is attached to avoid abandonment under 35 U.S.C. § 133 (extensions of time may be obtained under the provisions of 37 CFR 1.136(a)).

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

- ☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). If the effective filing date is on or after July 1, 1998, see the final rulemaking notice published at 63 FR 29620 (June 1, 1998) and 1211 OG 82 (June 23, 1998).
- ☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).
- ☐ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).
- ☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked-up "Raw Sequence Listing."
- ☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).
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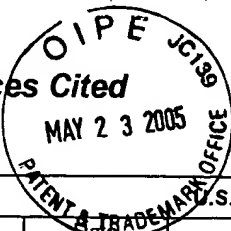
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	U	Brinkmann et al., PNAS USA, 88:8616-8620, 1991
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4/17/2005

B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice

(monoclonal antibody B3/single-chain antigen-binding protein/*Pseudomonas* exotoxin/cancer therapy)

ULRICH BRINKMANN, LEE H. PAI, DAVID J. FITZGERALD, MARK WILLINGHAM, AND IRA PASTAN*

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Contributed by Ira Pastan, June 17, 1991

ABSTRACT The genes encoding the heavy- and light-chain Fv regions of the monoclonal murine antibody B3, which recognizes a carbohydrate antigen on the surface of many human carcinomas, were cloned by PCR techniques and used to generate single-chain immunotoxins containing *Pseudomonas* exotoxin (PE). The light and heavy chains were connected by a flexible linker to form a single-chain antigen-binding protein, B3(Fv), which was in turn fused to truncated forms of PE lacking the cell-binding domain. The single-chain Fv and two different B3(Fv) immunotoxins, B3(Fv)-PE40 and B3(Fv)-PE38KDEL, were expressed in *Escherichia coli* and the single-chain immunotoxins were purified to near homogeneity. Both recombinant immunotoxins were shown to be cytotoxic specifically to carcinoma cell lines that express the B3 antigen on their surface; B3(Fv)-PE38KDEL was significantly more active. Furthermore, intravenous administration of B3(Fv)-PE38KDEL caused complete regression of human epidermoid carcinomas growing subcutaneously in immunodeficient mice.

The monoclonal antibody (mAb) B3 is a recently isolated murine antibody directed against a carbohydrate antigen in the Le^y family that is found on the surface of many mucinous carcinomas of the colon, stomach, ovaries, breast, and lung as well as some epidermal carcinomas (1). Because it reacts with only a limited number of normal tissues, mAb B3 is an ideal candidate for the treatment and diagnosis of cancer. To make a cytotoxic agent, mAb B3 was chemically coupled to two different forms of *Pseudomonas* exotoxin (PE). One of these was the full-length toxin (PE) and the other was a truncated derivative (PE40) (2). Both of these immunotoxins were cytotoxic selectively to tumor cells that contained the B3 antigen on their surface and caused complete tumor regression in mice bearing human tumor xenografts (3). Although this first-generation immunotoxin has properties that indicate it should be developed further as a drug for the treatment of cancer, immunotoxins made by chemical conjugation methods have several undesirable properties. One is that the chemical modifications can change the antibody and affect its binding to antigen. A second is that the purified immunotoxins are a heterogeneous mixture of antibody-toxin molecules connected to each other via different positions on the antibody and the toxin. Thus, PE can be coupled to either the light or the heavy chain of the antibody and to different positions on each of these chains. Immunotoxins of the second generation are made as recombinant antibody Fv-fusion proteins in bacteria. It has been shown (4, 5) that single-chain antigen-binding proteins (scABs, scFvs) made from the Fv portions of the heavy and light chain of antibodies held together by a polypeptide linker can have the same binding properties as their full-length two-chain counterparts. Furthermore, fusion proteins composed of scABs

linked to toxins retain the binding capacity of the scAB as well as the activity of the toxin (6-9).

Potent single-chain immunotoxins have been made previously by fusing the Fv domains of antibodies directed at the interleukin 2 receptor (6, 7) or at the transferrin receptor (8) to truncated forms of PE or diphtheria toxin (10). Receptors often make good immunotoxin targets because they are cell surface proteins that can be rapidly internalized, and toxins must be internalized to kill cells. Here we describe the cloning of DNA fragments that encode the Fv region of mAb B3, which is directed against a carcinoma-associated cell surface antigen, and the construction of plasmids for expression in *Escherichia coli* of the B3 single-chain antibody, B3(Fv), fused to two different truncated PE molecules. Further we show that these recombinant B3(Fv) immunotoxins are cytotoxic selectively to cultured human tumor cells bearing the B3 antigen, and they cause complete regression of human tumors growing in immunodeficient mice.

MATERIALS AND METHODS

Cloning of DNA Fragments Encoding the Heavy and Light Fv Region of mAb B3. Cloning experiments and propagation of plasmids were carried out generally in *E. coli* HB101 (11). DNA fragments encoding the Fv portions of the heavy and light chains of mAb B3 were obtained by PCR amplification of single-stranded DNA, which was synthesized by random-primed reverse transcription of mRNA from a mAb B3-producing hybridoma cell line. PCR (12) was performed with the Perkin-Elmer GeneAmp kit and a Perkin-Elmer/Cetus thermocycler, under conditions as described (9). For amplification of the heavy chain Fv coding region we chose the primer pair B3-H1 (5'-TAAGTGGATCCGTCATATG-GATGTGAAGCTGGTGGAGTCTGG-3') and B3-H2 (5'-TGGATAGACTGATGGGATCCGCCTCCGCCTGAG-GAGAC-3'), and for the light chain we chose B3-L1 (5'-GTCTCCAAGCTTGGGATCCGGTGGTGGCGGATCTGGAGGTGGCGGAAGCGATGTGCTGACCCAGTCTCC-3') and B3-L2 (5'-AGTTGGTGCAGCATC-AAAAGCTTT^GATCTCCAGCTT^GGT^GCC-3'). The recognition sequences for *Nde*I, *Hind*III, and *Bam*HI are underlined. These oligonucleotides have at their 3' end constant sequences that occur at the beginning and end of mouse Fv DNA. At their 5' end are restriction endonuclease recognition sites (*Nde*I, *Bam*HI, *Hind*III) for cloning of the PCR products as shown in Fig. 1. The products of the amplifications of heavy- and light-chain Fv DNA fragments were identified by agarose gel electrophoresis to be DNA fragments 350 to 400 bp long. They were purified from gels, cut with *Bam*HI or *Hind*III (Fig. 1), and, after purification on a second gel, ligated with *Hind*III- or *Bam*HI-linearized and

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Abbreviations: mAb, monoclonal antibody; PE, *Pseudomonas* exotoxin.

*To whom reprint requests should be addressed.

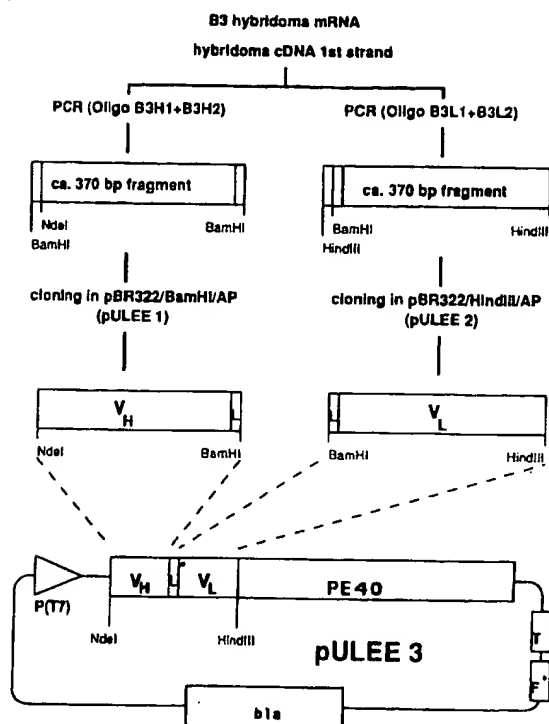


Fig. 1. Strategy for the cloning of the heavy- and light-chain Fv genes of mAb B3 and construction of plasmids for expression of B3(Fv) immunotoxins. The cloning strategy is a variation of that previously described (9). The plasmid pVC38H, which is used as a vector for construction of immunotoxins from heavy- and light-chain Fv regions (V_H and V_L , respectively), contains an *Nde*I and a *Hind*III recognition sequence preceding the PE40 gene (9). The sequences of the PCR primers are shown in *Materials and Methods*. The * indicates a PCR-derived mutation that was repaired by site-directed mutagenesis; L indicates the region encoding the (Gly₄Ser)₁ linker; bp, base pair; AP, dephosphorylation with alkaline phosphatase.

dephosphorylated pBR322 vector (13). The nucleotide sequence of the light- or heavy-chain Fv coding region of mAb B3 was determined from double-stranded plasmid DNA by using sequencing primers (New England Biolabs) adjacent to the *Bam*HI or *Hind*III site of pBR322 and a T7 DNA polymerase sequencing reagent kit (United States Biochemical).

Construction of Plasmids for Expression of B3(Fv) and B3(Fv)-Immunotoxins. The expression plasmid pVC38H contains the gene for the immunotoxin TGF α -PE40 (TGF α , transforming growth factor α) under control of the T7 promoter (9), the T Φ transcription terminator at the 3' end of the PE40 coding region, and the single-strand replication origin, F⁺, to generate single-stranded phage DNA by cotransfection with (M13) helper phages, if desired, to create derivatives of the plasmid by site-directed mutagenesis. The TGF α coding region in pVC38H has an *Nde*I recognition site at the 5' end and a *Hind*III site at the point of connection to the DNA encoding PE40. To create a plasmid for expression of the immunotoxin B3(Fv)-PE40 (pULEE3), the TGF α gene was removed and replaced by the B3(Fv) gene in a three-fragment ligation, using an *Nde*I/*Bam*HI fragment of the heavy-chain coding region and the *Bam*HI/*Hind*III fragment encoding the light chain Fv (Fig. 1). Because sequence analysis showed a mutation (deletion and frameshift) at the 5' end of the light-chain Fv gene due to a sequence repetition in the PCR primer annealing region, site-directed mutagenesis

was performed, using uridine-containing single-stranded DNA obtained by the Kunkel method (14) from the F⁺ origin of pULEE3 as the mutagenesis template. In the resulting plasmid (pULI1), the correct amino end of the B3 light chain established by partial protein sequencing of mAb B3 is reconstructed. To make another B3(Fv) immunotoxin, B3(Fv)-PE38KDEL, the PE40-encoding region was removed from pULI1 from the *Hind*III site to an *Eco*RI site positioned just beyond the PE40 gene and was replaced by a *Hind*III/*Eco*RI fragment from pRK79K (R. Kreitman and I.P., unpublished results) encoding the PE variant PE38KDEL, which has domain I (amino acids 1–252) and part of domain Ib (amino acids 365–380) deleted and also contains an altered carboxyl-terminal sequence, KDEL (15). The expression plasmid pULI4 for production of B3(Fv) was constructed by removal of the light-chain and PE40-encoding region from pULI1 from *Bam*HI to *Eco*RI replacing it with a PCR fragment obtained by amplification of the light-chain Fv-encoding sequence with the primer pair B3-L3 + B3-L4. The primer B3-L3 (5'-TTGGGGATCCGGTGGTGGCGGATC-TGGA-3') is similar to B3-L1, used for cloning of light-chain Fv from cDNA, and B3-L4 (5'-AGCGGGAATTCATTATT-TAATTCCAGCTTTGTCCCCGAGC-3') is identical to B3-L2 in the 3' part for priming the PCR, but at the 5' end the *Hind*III site for fusion to PE sequences is replaced by translation stop codons followed by an *Eco*RI recognition sequence.

Expression and Purification of Recombinant B3(Fv)-Immunotoxins. Plasmids were transformed in the expression host *E. coli* BL21(ADE3) (16). The bacteria were grown in "superbroth" containing 2% glucose, 0.05% MgSO₄, and ampicillin at 100 μ g/ml, induced in the logarithmic phase at OD₆₀₀ of 3 with 1 mM isopropyl β -D-thiogalactoside, and harvested 90 min later. The recombinant protein was purified and analyzed as previously described (6, 7). Protein concentrations were determined by Bradford assay (17).

Cytotoxic Activity of Chemically Linked and Recombinant Immunotoxins. Assays measuring inhibition of protein synthesis were previously described (6, 7). All assays were performed in 96-well plates, each well containing 1.6×10^4 cells in 200 μ l of medium. For competition assays designed to prove the specificity of the recombinant immunotoxins, we changed the medium and added 50 μ g of antibody per well 15 min prior to the addition of the immunotoxin.

Assay of Blood Levels of B3(Fv)-PE38KDEL in Mice. Six-week-old (19- to 20-g) female BALB/c mice were injected with 10 μ g of B3(Fv)-PE38KDEL in the tail vein. Blood was drawn at various time intervals and the level of the immunotoxin was measured by incubating serum with A431 cells and measuring inhibition of protein synthesis. A standard curve was made with pure B3(Fv)-PE38KDEL and the blood level of immunotoxin was calculated by using this curve.

Antitumor Activity of B3(Fv)-PE38KDEL in Nude Mice Bearing a Human Epidermoid Carcinoma. A431 carcinoma cells (3×10^6) were injected subcutaneously on day 0 into female nude mice (4–6 weeks old, 18–20 g). Mice with 5 mm \times 5 mm tumors, which usually developed by day 4, were treated with B3(Fv)-PE38KDEL or as a control with mAb B3 or anti-Tac(Fv)-PE38KDEL (6). Usually six injections were given at 12-hr intervals into the tail vein, starting 4 days after tumor implantation. Each treatment group consisted of five animals. The volume of the tumor (cm³) was calculated by length \times width² \times 0.4.

RESULTS

Cloning and Expression of cDNAs Encoding the Variable Regions of B3 as a Single-Chain Fv and as a Single-Chain Immunotoxin. To obtain DNA fragments encoding the Fv region of the light and heavy chain of mAb B3, mRNA was

prepared from the B3 hybridoma cell line (1) and transcribed into single-stranded DNA with reverse transcriptase (Fig. 1). The Fv-encoding regions were subsequently amplified by the PCR, using primers containing consensus sequences present in the conserved regions at the 5' and 3' ends of the Fv domain of mouse heavy and light chains (see *Materials and Methods*). The amplified fragments of the expected size (370–400 bp) were then cloned in pBR322, sequenced, and subcloned in plasmids for expression of either the B3(Fv) alone or B3(Fv) fusions to two different forms of truncated PE. One is PE40, which contains the translocating and ADP-ribosylating domains of PE (amino acids 253–613), but not the cell-binding domain (amino acids 1–252). The other is PE38KDEL, which is derived from PE40 but has a deletion

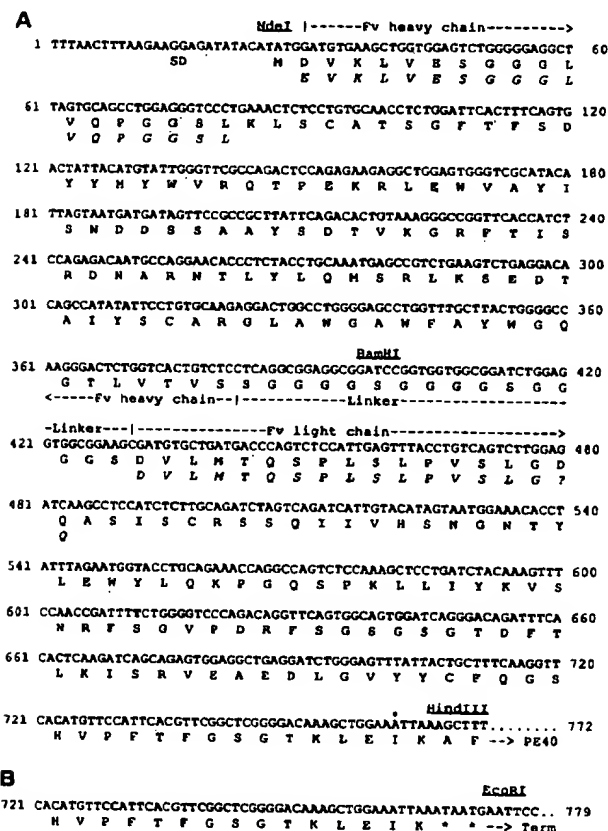


FIG. 2. Nucleotide sequences encoding the heavy- and light-chain Fv region of mAb B3. (A) The heavy chain Fv coding region extends from position 30 to 383, the light-chain Fv gene from position 433 to 767, and the linker from 384 to 432. The deduced amino acid sequence (one-letter symbols) is shown in roman letters; below in italic letters is the protein sequence determined by Edman sequencing of the antibody. The first amino acid encoded by the cloned heavy-chain Fv gene is Asp instead of Glu due to the oligonucleotide primer used. Position 456–465 is the region where the PCR cloning artifact was repaired. This sequence encodes the same amino acids as the original B3 light-chain gene but uses other codons. Homology comparisons to the known nucleotide sequence of PAC1 immunoglobulin κ chain (19), which is most similar to the B3 light chain (see *Discussion*), indicates that the original sequence was most probably CTCTCCCTG instead of TTGAGTTTA. Thus the natural B3 light chain gene has a sequence repetition between positions 445 and 465 5'-(CCAGTCTTCC)ACTCTCC-3' which is seen by comparing the sequences in each of the overlapping brackets and which is responsible for the incorrect primer annealing in PCR. (B) Sequence at the 3' end of the light chain for expression of the single chain B3(Fv) alone. SD, Shine-Dalgarno consensus sequence; *, translation stop signal; Term, transcription terminator.



FIG. 3. SDS/PAGE (20) of recombinant B3(Fv) and B3(Fv) immunotoxins from bacterial inclusion bodies on a 12.5% polyacrylamide gel. Lane a, total cell protein of induced bacteria producing single-chain B3(Fv); lane b, total protein of cells producing B3(Fv)-PE40; lanes c and d, supernatant of sonicated cells producing B3(Fv) (c) or B3(Fv)-PE40 (d); lane e, inclusion bodies containing B3(Fv); lane f, B3(Fv)-PE40 inclusion bodies; lane g, purified B3(Fv)-PE40 protein after gel filtration. MW, molecular weight standards, indicated on the left $\times 10^{-3}$.

of amino acids 365–380, which removes a disulfide bond (18), and a change in the carboxyl end of PE (KDEL), which increases cytotoxic activity (15).

A summary of the cloning strategy and the structure of the B3(Fv)-PE40 gene is shown in Fig. 1. Sequence analysis of the PCR products obtained after the first cloning step in pBR322 indicated that a frameshift deletion had occurred at the beginning of the light chain, resulting in the deletion of seven nucleotides. This artifact is likely due to a sequence repetition at the beginning of the light chain where the PCR primer anneals (see Fig. 2 for details). This sequence repetition, which is a conserved region in the particular class of

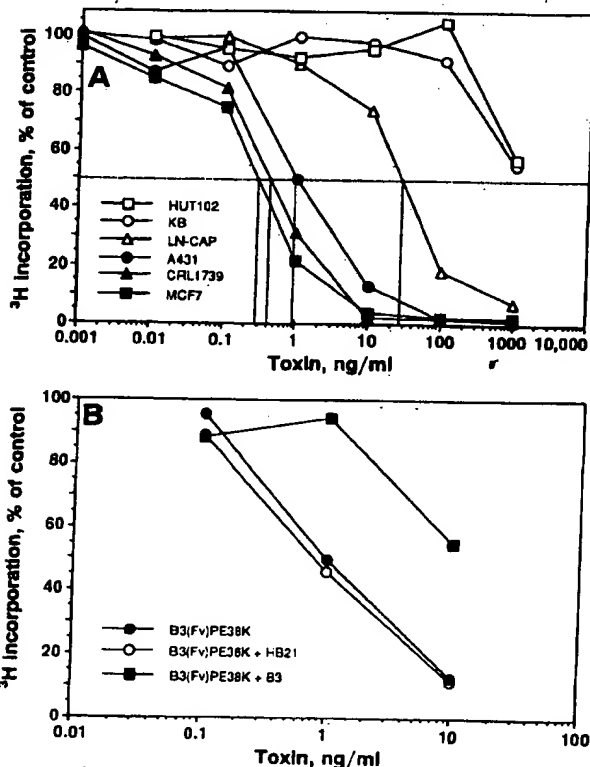


FIG. 4. (A) Toxicity of B3(Fv)-PE38KDEL for various cell lines. (B) mAb B3 inhibition of the cytotoxicity of B3(Fv)-PE38KDEL for A431 cells.

Table 1. Activities of B3 immunotoxins on various cell lines

Cell line	Cancer type	B3 antigen	Cytotoxicity (ID ₅₀ , ng/ml (pM))		
			B3(Fv)-PE40	B3(Fv)-PE38KDEL	B3-LysPE40
MCF7	Breast	++	3 (50)	0.2 (3.2)	3 (16)
CRL1739	Gastric	++	3 (50)	0.3 (5)	3 (16)
A431	Epidermoid vulva	+	3 (50)	0.8 (13)	8 (42)
LNCaP	Prostate	+	40 (1330)	20 (325)	85 (460)
KB3-1	Epidermoid cervix	-	>1000	>1000	>1000
HUT102	Adult T-cell leukemia	-	>1000	>1000	>1000

light chains to which B3 belongs, could make it difficult to clone other light-chain Fv sequences of this class by using "consensus" PCR primers. We repaired this deletion by site-directed mutagenesis, using as our guide the amino-terminal sequence of the B3 light chain, which was determined by Edman degradation to be VLMTQSPLSLPVSLGXQ. The heavy-chain sequence was also determined by Edman degradation and found to be EVKLVESGGGLVQPGGSL. Comparison of these amino acid sequences with the sequences deduced from the nucleotide sequences of the cloned Fv DNA segments indicated that the clones isolated encoded the light- and heavy-chain variable regions of mAb B3 (Fig. 2).

Expression of B3(Fv)-PE40 and B3(Fv)-PE38KDEL was carried out as described in *Materials and Methods*. About 30% of the total protein of the induced cultures was the recombinant expression product, deposited in inclusion bodies. The purified inclusion bodies contained almost pure recombinant protein, which had the expected molecular weight of about 67,000 for a single-chain immunotoxin (Fig. 3). These immunotoxin molecules were solubilized, refolded, and purified; their purity is shown in Fig. 3.

Cytotoxicity of B3(Fv)-PE40 and B3(Fv)-PE38KDEL. Two different recombinant B3(Fv) immunotoxins were analyzed for their ability to inhibit protein synthesis. As shown in Fig. 4 and in Table 1, the recombinant single-chain immunotoxins inhibited protein synthesis in cells expressing the B3 antigen but not in nonexpressing cells, similar to the previously described results with a chemical conjugate of B3 with a truncated form of PE (2). The relative potencies of the chemical conjugate and the single-chain immunotoxins were about the same on the four antigen-positive cell lines MCF-7, CRL 1739, A431, and LNCaP. The most active agent was B3(Fv)-PE38KDEL. To analyze whether the cytotoxicity of B3(Fv)-immunotoxins was specific, competition experiments

were carried out with an excess of mAb B3. The data in Fig. 4B show that the intoxication of A431 carcinoma cells by B3(Fv)-PE38KDEL is due to the specific binding to the B3 antigen, since its cytotoxicity was blocked by excess mAb B3 but not by mAb HB21, which recognizes the transferrin receptor on these cells (21). A large excess of mAb B3 is necessary for reversal of cytotoxicity, probably because there is a large amount of the B3 antigen on the surface of A431 cells (1, 3).

Antitumor Effects of B3(Fv)-PE38KDEL in Mice. Because B3(Fv)-PE38KDEL was the most active agent in cell culture, it was tested against subcutaneous tumor xenografts in nude mice. To do this, A431 cells were implanted subcutaneously; 4 days later tumors had developed. Because the lifetime of B3(Fv)-PE38KDEL in the circulation of the mice was observed to be only 15–20 min (Fig. 5), we chose to inject the single-chain immunotoxin twice daily for 3 successive days. As shown in Fig. 6, injection of 2.5, 5, or 10 μ g twice daily produced complete tumor regression. Partial regression was observed when only 0.5 μ g was injected. No toxicity was observed at these doses. In addition, when mice with large tumors, about 1 cm in diameter and containing about 5×10^8 cells, were treated with 5 μ g twice a day for 4 days, complete regression rapidly occurred (Fig. 6D). Regression of MCF-7 tumors (breast carcinoma) also was observed with 5 μ g of B3(Fv)-PE38KDEL twice daily. In control experiments, mice were treated with either mAb B3 or anti-Tac(Fv)-PE38KDEL, which is cytotoxic for cells with interleu-

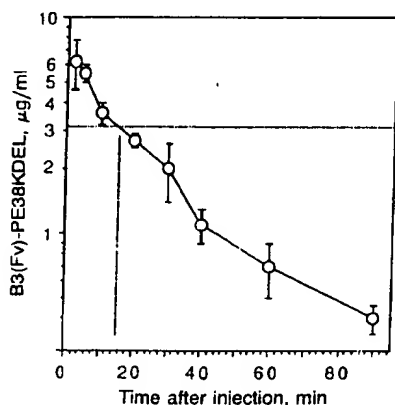


FIG. 5. Blood levels of B3(Fv)-PE38KDEL in mice. BALB/c mice were injected i.v. with 10 μ g of B3(Fv)-PE38KDEL and immunotoxin concentrations were measured at different time periods. Bars indicate SD.

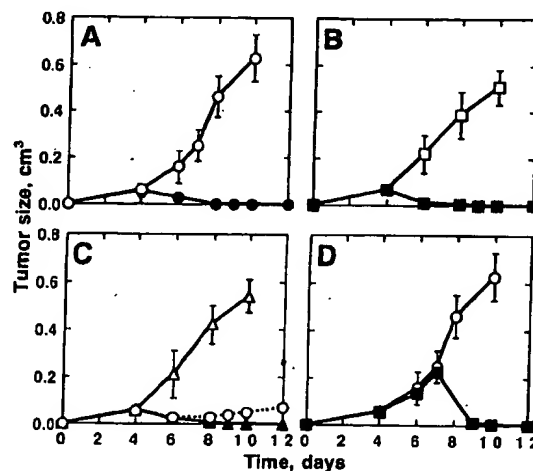


FIG. 6. Effect of B3(Fv)-PE38KDEL on the growth of A431 tumors in nude mice. Mice were injected with 3×10^6 A431 cells on day 0 and treated beginning on day 4 with i.v. injections every 12 hr for 3 days (six injections, total). (A) \circ , Untreated; \bullet , 10 μ g of B3(Fv)-PE38KDEL. (B) \square , 2.5 μ g of B3(Fv)-PE38KDEL; \blacksquare , 5 μ g of B3(Fv)-PE38KDEL. (C) \triangle , 2.5 μ g of anti-Tac(Fv)-PE38KDEL; \blacktriangle , 2.5 μ g of B3(Fv)-PE38KDEL; \circ , 0.5 μ g of B3(Fv)-PE38KDEL. (D) Treatment began on day 7 with i.v. injections every 12 hr for 4 days (eight injections, total). \circ , Untreated; \blacksquare , 5 μ g of B3(Fv)-PE38KDEL. Bars indicate SD.

Table 2. Structure and activity of B3 immunotoxins on A431 cells

Immunotoxin	Toxin part	Carboxyl terminus	Binding	ID ₅₀	
				ng/ml	pM
B3 chemical conjugate	PE40	REDLK	Bivalent	8.0	42
B3(Fv) fusion protein	PE40	REDLK	Monovalent	3.0	50
B3(Fv) fusion protein	PE38	KDEL	Monovalent	0.8	13

kin 2 receptors but not for A431 cells (6), and no antitumor effect was observed.

DISCUSSION

We have cloned DNA sequences encoding the heavy- and light-chain Fv domains of the murine mAb B3. This mAb recognizes an antigen present on many carcinoma cells, and it may be useful for the treatment of various types of cancers (1, 3). The heavy- and light-chain regions of mAb B3 were connected by a flexible linker (Gly₄Ser)₃, which started at the carboxyl end of the heavy-chain Fv domain and ended at the amino terminus of the light-chain Fv domain. The resulting gene encodes the B3(Fv) domain in the form of a single-chain antigen-binding protein. We then fused this B3(Fv) gene to sequences encoding two different truncated forms of the PE molecule to obtain single-chain B3(Fv) immunotoxins. These recombinant immunotoxins were shown to kill carcinoma cells containing the B3 antigen without affecting control cells. The cytotoxicity of the recombinant B3(Fv)-PE40 was similar to a chemical conjugate of B3 and PE40 (B3-LysPE40) (3), but B3(Fv)-PE38KDEL was considerably more active. Furthermore, B3(Fv)-PE38KDEL caused complete regression of A431 tumors grown in immunodeficient mice. This makes the B3(Fv)-derived single-chain immunotoxins a promising alternative to B3 chemical conjugates and a possible second-generation immunotoxin for the treatment of solid tumors.

The authenticity of the cloned DNA fragments was proven by comparing the amino-terminal protein sequences of the B3 heavy and light chains with the amino acid sequences deduced from the reading frames of the cloned genes (Fig. 2). The DNA sequence data further showed that the B3 heavy chain is a member of the mouse class III (A) heavy-chain group (22) and that it is 90% identical to the heavy chain of mAb 50.1, which, like mAb B3, recognizes a β -galactan-containing carbohydrate epitope (23). The light chain is a member of the κ class II group (22) and is 96% identical to the κ light chain of an antibody to the platelet fibrinogen receptor (19).

The analysis of the cytotoxicity of B3-immunotoxins showed the same sensitivity pattern of different cell lines towards the recombinant immunotoxins as towards the chemical conjugates. All these immunotoxins were very cytotoxic to carcinoma cells that express the B3 antigen on their surface, including MCF7 (breast), A431 (epidermoid), CRL1739 (gastric), and LNCaP (prostate). Also, the recombinant single chain B3-Fv immunotoxins did not affect B3 antigen-negative control cells. The cytotoxicity of the recombinant B3(Fv)-PE40 (ID₅₀ = 50 pM; 3.0 ng/ml) was similar to that of the chemically linked B3-immunoconjugate (ID₅₀ = 42 pM; 8 ng/ml), whereas B3(Fv)-PE38KDEL was much more active than the chemical conjugate (ID₅₀ = 13 pM; 0.8 ng/ml). This is despite the fact that the single-chain immunotoxins possess only one antigen-binding site per molecule and the chemical conjugate has two (Table 2). B3(Fv)-PE38LDEL has two features that distinguish it from B3(Fv)-PE40. One is

that a portion of domain Ib encompassing amino acids 365–380 is deleted. This removes a disulfide bond formed between cysteine residues at positions 372 and 379, which might form disulfide bonds with other cysteines during the renaturation procedure and result in inactive chimeric toxins. The second feature is that the carboxyl terminus of the toxin is changed from the original sequence REDLK to KDEL. This type of change often produces a 2- to 3-fold increase in the activity of chimeric toxins (24).

B3(Fv)-PE38KDEL was tested for its antitumor effect in nude mice bearing A431 tumors. Complete regression of tumors was observed when mice received 2.5, 5, or 10 μ g of the chimeric toxin twice daily for 3 days, despite the fact that B3(Fv)-PE38KDEL has a short lifetime (15–20 min) in the circulation. B3(Fv)-PE38KDEL also produced complete regression of tumors about 1 cm in diameter. Previously we found it required daily injections of 25 μ g of a chemical conjugate composed of B3 and PE40 (see Table 2) to produce complete regression of small tumors and partial regression of large tumors despite the fact that the chemical conjugate has a much longer lifetime in the blood (4 hr). The recombinant molecule probably has a higher antitumor activity in the mouse model because of its small size, which allows better access to tumor cells.

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